Appendix B1

Protocol for CV1 + hAR + Luciferase Assay

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6 cm dish CV1 LUCIFERASE ASSAY (hAR) Revised 1-25-02

Monday

1. recount cells: best to count 50-100 cells per 5x5 in hymocytometer, count 2 5x5 grids and average, count x 10^4 = cells/ml
2. plate 0.42 x 10^6 CV1 cells/6 cm dish by preparing large mix of cells and media so 4 ml media/plate containing 5% bovine calf serum, DMEM-H/20 mM Hepes (2 M Hepes stock, pH 7.2, filter), penicillin and streptomycin, 2 mM L-glutamine, spread cells evenly. Cells usually added from master mix; swirl often while adding cells to the plates.

Tuesday

prepare $CaPO_4$ precipitates for groups of up to 6 plates using freshly made solutions: for large assays of same DNA, pool the precipitates before adding to the plates.

(a) make 2 M $CaCl_2$: 2.94 g $CaCl_2.2H_20$ bring to 10 ml with sterile autoclaved dH_2O, filter sterilize

(b) make 2X HBS: 500 ml 8.2 g NaCl
12.5 ml 2 M Hepes
0.2 g Na_2HPO_4-$7H_20$
bring to 500 ml with sterile autoclaved dH_20 from TC room, pH with 5 N NaOH (takes 150-200 µl), pH to 7.11-7.14, sterile filter, make 27.5 ml aliquots (25 ml needed for 100 6 cm plates), store frozen at –20°C

[for 50 ml 2XHBS: 14 ml 1 M NaCl, 0.25 ml 2 M Hepes Na salt, 750 µl 0.1 M Na_2HPO_4, bring to 45 ml with sterile ddH_20 (use sterile autoclaved water), add about 45 µl 5 M NaOH, pH to 7.11-7.14, filter sterilize, store pH electrode in pH 7 buffer, NOT H_2O]

(c) prepare DNA one or more days before assay
make dilutions of DNA stocks so additions are ~3-10 µl
add expression and reporter DNA to bottom of 14 ml polystyrene round-bottom (17x100 mm) Falcon tubes, store frozen –20˚C

50 ng pCMVhAR (or 10 ng pCMVhAR1-660 (ABC))
5 µg MMTV-luciferase
[For PSA-luciferase use 5 µg reporter/dish, 100 ng pCMVhAR/dish]

(d) per 6 cm dish, add to tubes containing DNA:
210 µl sterile H_20
30 µl 2 M $CaCl_2$ (final 0.125 M $CaCl_2$)
240 µl 2X HBS, vortex briefly, let sit 30 min at RT
vortex briefly, add 475 µl of mix per well, return plates to incubator, incubate 4 h

example for 6 dishes:

DNA	H_20	2 M $CaCl_2$	2XHBS
0.3 µg pCMVhAR 30 µg MMTV-Luc	1.26 ml (2 x 630 µl)	180 µl	1.44 ml (2 x 720 µl)

(e) aspirate plates, add 1.5 ml glycerol shock medium, incubate 3 min RT, aspirate, wash 4 ml PBS, aspirate, add 4 ml serum free, phenol red free DMEM-H, Hepes, P/S, glutamine ± hormone, return to incubator for overnight

Glycerol Shock Medium: use 5% DMEM-H red
dishes x 1.5 ml/dish = total volume (make extra)
total volume x 15% = amount of glycerol
total volume – amount of glycerol = amount of media to add with glycerol

Wednesday aspirate media, add 4 ml fresh phenol red-free, serum-free DMEM-H, P/S, Hepes, glutamine ± hormone, add DHT to stock media as needed and add to plates, incubate overnight 37˚C

Thursday remove media, wash with 4 ml PBS, aspirate to dry; add 0.5 ml/plate lysis buffer; rock plates 20-30 min, not much longer at RT. Transfer 100 µl from each well to 96 well Nunc flat bottom standard nontreated white plate. Read on LumiStar automated luminometer that injects 100 µl luciferin stock and 100 µl reading buffer

Lysis buffer: 2 mM EDTA, 1% Triton X-100, 25 mM Trizma (Tris base) phosphate, pH 7.8

D-Luciferin: prepare 1 mM D-luciferin (K+ salt, MW 318.41) in dH_2O store in 10 ml aliquots at -20°C covered with foil (D-luciferin is light sensitive) use 100 µl/sample, save extra at -20°C, Na+ salt sometimes turns yellow but is probably still good, pH of H_2O might be off, better to use K+ salt) (from Analytical Luminescence)

Reading buffer: The optimal pH for the reaction is pH 7.8 cold; if glycylglycine and ATP are carefully pH cold, then the final will be pH 7.8

Stock	Amount to 20 ml final	Amount to 100 ml final	Final conc
0.5 M glycylglycine, pH 7.8 cold	1 ml	5 ml	25 mM
1 M $MgCl_2$	300 µl	1.5 ml	15 mM
100 mM ATP in dH_2O	1 ml	5 ml	5 mM
(bring to pH 7.8 cold with 1 M NaOH, CRITICAL, store -80°C 1 ml aliquots)			
Sigma - tissue culture grade			
50 mg/ml BSA dH_2O	200 µl	1 ml	0.5 mg/ml
dH_2O	17.5 ml	87.5 ml	
need 40 ml for 100 plates			